

What is claimed is:

1. A method for producing a library of mutagenized polynucleotide from a target sequence comprising:

5 (a) taking a sample comprising

(i) a target sequence including a section to be mutagenized,

10 (ii) a library of first primers where the first primers include a first fixed sequence and a first unknown sequence 3' to the first fixed sequence, the first unknown sequence varying within the library of first primers, and

15 (iii) a library of second primers where the second primer include a second fixed sequence that differs from the first fixed sequence, and a second unknown sequence 3' to the second fixed sequence, the second unknown sequence varying within the library of second primers;

20 (b) performing one or more cycles of primer extension amplification on the sample in the presence of at least one polymerase such that a member of the library of the first primers is extended relative to the target sequence; and

25 (c) performing one or more additional cycles of primer extension amplification on the sample such that a member of the library of the second primers is extended relative to the first primer that was extended in step (b) to form the library of mutagenized polynucleotides.

2. The method according to claim 1 wherein at least one of the first or second unknown sequences is unknown at the time of primer extension amplification.

3. The method according to claim 1 wherein the target sequence has a sequence which is at least partially unknown at the time of primer extension amplification.
- 5 4. The method according to claim 1, wherein the target sequence has a sequence which is the CDR of an antibody.
5. The method according to claim 1, wherein the target sequence has a sequence encoding a single-chain antibody.
- 10 6. The method according to claim 1 wherein the first and second fixed sequences include at least one restriction site.
- 15 7. The method according to claim 1 wherein one of the fixed sequence of the first and second primers includes an ATG or a GTA sequence and the fixed sequence of the other primer includes a sequence encoding one or more translation stop codons.
- 20 8. The method according to claim 1 wherein the first or second primers include one or more inosines at the 3' end penultimate and ultimate positions.
- 25 9. The method according to claim 1 wherein the length of the first and second primers is between 10 and 80 nucleotides.
10. The method according to claim 1 wherein the first or second unknown sequence has a length between 3 and 70 nucleotides.
- 30 11. The method according to claim 1 wherein the first or second unknown sequence has a length between 4 and 50 nucleotides.

12. The method according to claim 1 wherein the first or second unknown sequence has a length between 5 and 20 nucleotides.
13. The method according to claim 1 wherein the first or second
5 unknown sequence further includes a sequence encoding one or more specific amino acid residues.
14. The method according to claim 13 wherein the one or more
10 specific amino acid residues are conserved amino acid residues of the protein encoded by the target sequence.
15. The method according to claim 1 wherein at least a portion of the multiple cycles of primer extension polymerase amplification is performed such that extension by the polymerase is at least partially
15 performed at a temperature below 70°C for at least 30 sec.
16. The method according to claim 1 wherein at least a portion of the multiple cycles of primer extension polymerase amplification is performed such that extension by the polymerase is at least partially
20 performed at a temperature below 60°C for at least 30 sec.
17. The method according to claim 1 wherein at least a portion of the multiple cycles of primer extension polymerase amplification is performed such that extension by the polymerase is at least partially
25 performed at a temperature below 50°C for at least 30 sec.
18. The method according to claim 1 wherein at least a portion of the one or more cycles of primer extension polymerase amplification is performed such that extension by the polymerase is at least partially
30 performed by heating the amplification reaction mixture from

temperature of between 30°C to 50°C to a temperature between 65°C to 75°C over the course of at least 30 sec.

5 19. A method for producing mutagenized polynucleotide from a target sequence comprising:

(a) taking a sample comprising

(i) a target sequence including a section to be mutagenized,

10 (ii) a first primer where the first primer includes a first fixed sequence and a first unknown sequence 3' to the first fixed sequence, and

(iii) a second primer where the second primer includes a second fixed sequence that differs from the first fixed sequence, and a second unknown sequence 3' to the second fixed sequence;

15 (b) performing one or more cycles of primer extension amplification on the sample in the presence of at least one polymerase such that the first primer is extended relative to the target sequence; and

(c) performing one or more additional cycles of primer extension amplification on the sample such that the second primer is extended
20 relative to the first primer that was extended in step (b) to form the mutagenized polynucleotide.

25 20. The method according to claim 19 wherein the first or second unknown sequence is unknown at the time of primer extension amplification.

30 21. The method according to claim 19 wherein the target sequence has a sequence which is at least partially unknown at the time of primer extension amplification.

22. The method according to claim 19 wherein the first and second fixed sequences include at least one restriction site.
23. The method according to claim 19 wherein one of the fixed sequence of the first and second primers includes an ATG or a GTA sequence and the fixed sequence of the other primer includes a sequence encoding one or more translation stop codons.
24. The method according to claim 19 wherein the length of the first and second primers is between 10 and 80 nucleotides.
25. The method according to claim 19 wherein the first or second unknown sequence has a length between 3 and 70 nucleotides.
26. The method according to claim 19 wherein the first or second unknown sequence has a length between 4 and 50 nucleotides.
27. The method according to claim 19 wherein the first or second unknown sequence has a length between 5 and 20 nucleotides.
28. The method according to claim 19 wherein the first or second unknown sequence further includes a sequence encoding one or more specific amino acid residues.
29. The method according to claim 28 wherein the one or more specific amino acid residues are conserved amino acid residues of the protein encoded by the target sequence.
30. The method according to claim 19 wherein at least a portion of the multiple cycles of primer extension polymerase amplification is

performed such that extension by the polymerase is at least partially performed at a temperature below 70°C for at least 30 sec.

5 31. The method according to claim 19 wherein at least a portion of the multiple cycles of primer extension polymerase amplification is performed such that extension by the polymerase is at least partially performed at a temperature below 60°C for at least 30 sec.

10 32. The method according to claim 19 wherein at least a portion of the multiple cycles of primer extension polymerase amplification is performed such that extension by the polymerase is at least partially performed at a temperature below 50°C for at least 30 sec.

15 33. The method according to claim 19 wherein at least a portion of the one or more cycles of primer extension polymerase amplification is performed such that extension by the polymerase is at least partially performed by heating the amplification reaction mixture from temperature of between 30°C to 50°C to a temperature between 65°C to 75°C over the course of at least 30 sec.

20 34. A method for producing a library of mutagenized polynucleotides from a target sequence, comprising:

- 25 (a) taking a sample comprising
- (i) a target sequence including a section to be mutagenized,
 - (ii) a library of first primers where the first primers include a first fixed sequence and a first unknown sequence 3' to the first fixed sequence, the first unknown sequence varying within the library of first primers, and
 - 30 (iii) a library of second primers where the second primers include a fixed sequence that differs from the first fixed sequence;

(b) performing one or more cycles of primer extension amplification on the sample in the presence of at least one polymerase such that a member of the library of the first primers is extended relative to the target sequence; and

5 (c) performing one or more additional cycles of primer extension amplification on the sample such that a member of the library of the second primers is extended relative to the first primer that was extended in step (b) to form the library of mutagenized polynucleotides.

10 35. The method according to claim 34 wherein the unknown sequence is unknown at the time of primer extension amplification.

36. The method according to claim 34 wherein the target sequence has a sequence which is at least partially unknown at the time of primer extension amplification.

15 37. The method according to claim 34 wherein the target sequence has a sequence which is at least partially unknown at the time of primer extension amplification.

20 38. The method according to claim 34 wherein the target sequence has a sequence which is the CDR of an antibody.

25 39. The method according to claim 34 wherein the first and second fixed sequences include at least one restriction site.

40. The method according to claim 34 wherein the second fixed sequence includes a sequence that is substantially homologous to a portion of the target sequence.

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- 5 41. The method according to claim 34 wherein one of the fixed sequence of the first and second primers includes an ATG or a GTA sequence and the fixed sequence of the other primer includes a sequence encoding one or more translation stop codons.
42. The method according to claim 34 wherein the first or second primers include one or more inosines at the 3' end penultimate and ultimate positions.
- 10 43. The method according to claim 34 wherein the length of the first and second primers is between 10 and 80 nucleotides.
- 15 44. The method according to claim 34 wherein the unknown sequence has a length between 3 and 70 nucleotides.
45. The method according to claim 34 wherein the unknown sequence has a length between 4 and 50 nucleotides.
- 20 46. The method according to claim 34 wherein the unknown sequence has a length between 5 and 20 nucleotides.
- 25 47. The method according to claim 34 wherein the unknown sequence further includes a sequence encoding one or more specific amino acid residues.
- 30 48. The method according to claim 47 wherein the one or more specific amino acid residues are conserved amino acid residues of the protein encoded by the target sequence.
49. The method according to claim 34 wherein at least a portion of the multiple cycles of primer extension polymerase amplification is

performed such that extension by the polymerase is at least partially performed at a temperature below 70°C for at least 30 sec.

5 50. The method according to claim 34 wherein at least a portion of the multiple cycles of primer extension polymerase amplification is performed such that extension by the polymerase is at least partially performed at a temperature below 60°C for at least 30 sec.

10 51. The method according to claim 34 wherein at least a portion of the multiple cycles of primer extension polymerase amplification is performed such that extension by the polymerase is at least partially performed at a temperature below 50°C for at least 30 sec.

15 52. The method according to claim 34 wherein at least a portion of the one or more cycles of primer extension polymerase amplification is performed such that extension by the polymerase is at least partially performed by heating the amplification reaction mixture from temperature of between 30°C to 50°C to a temperature between 65°C to 75°C over the course of at least 30 sec.

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